Mapping the Apodemes of the Haltere Steering Muscles in *Drosophila melanogaster*

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May 5, 2021

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Abstract

Insects demonstrate some of the most masterful flying abilities in the animal kingdom, while also battling the physiological constraints of their small size and high wing-beat frequency. *Drosophila melanogaster*, in particular, are ideal organisms for the study of flight control due to their streamlined physiology and impressive maneuverability during flight. Like all Diptera, *Drosophila* have small appendages called halteres attached to their thoraxes where hindwings would typically be. The halteres beat antiphase to the wings and function like gyroscopes. They have also been shown to act as adjustable metronomes for flight control, dynamically adjusting the firing phase of the wing steering muscles. An important factor in understanding the flight control of *Drosophila* is how flies are able to change the trajectory of their halteres and transmit sensory and timing feedback to the wing steering system. Though the steering muscles of the halteres have been identified, researchers still do not understand what length changes these muscles undergo during flight and how they are recruited by motor neurons. In order to understand how *Drosophila* generate haltere trajectory changes, a thorough understanding of these steering muscles is required. This research sought to gain a clearer picture of the functioning of the haltere steering muscles and how they generate force by mapping the tendon-like structures called apodemes that attach to the steering muscles. To achieve this, I imaged and created confocal stacks of the haltere steering muscles and their apodemes in order to identify the number and location of the apodemes. I then imaged the apodemes during flight. My analysis showed that the basalar apodemes are fused to one another and that the largest and most prominent apodemes of the haltere are the fused basalar apodeme as well as the apodeme of the hIII2 axillary muscle. These findings indicate a particularly important role for these muscles in controlling haltere trajectory changes and, thus, in influencing flight. Furthermore, given that the

basalar apodemes are fused to one another, this finding also provides insight into how those two muscles are recruited in tandem by their corresponding motor neurons.

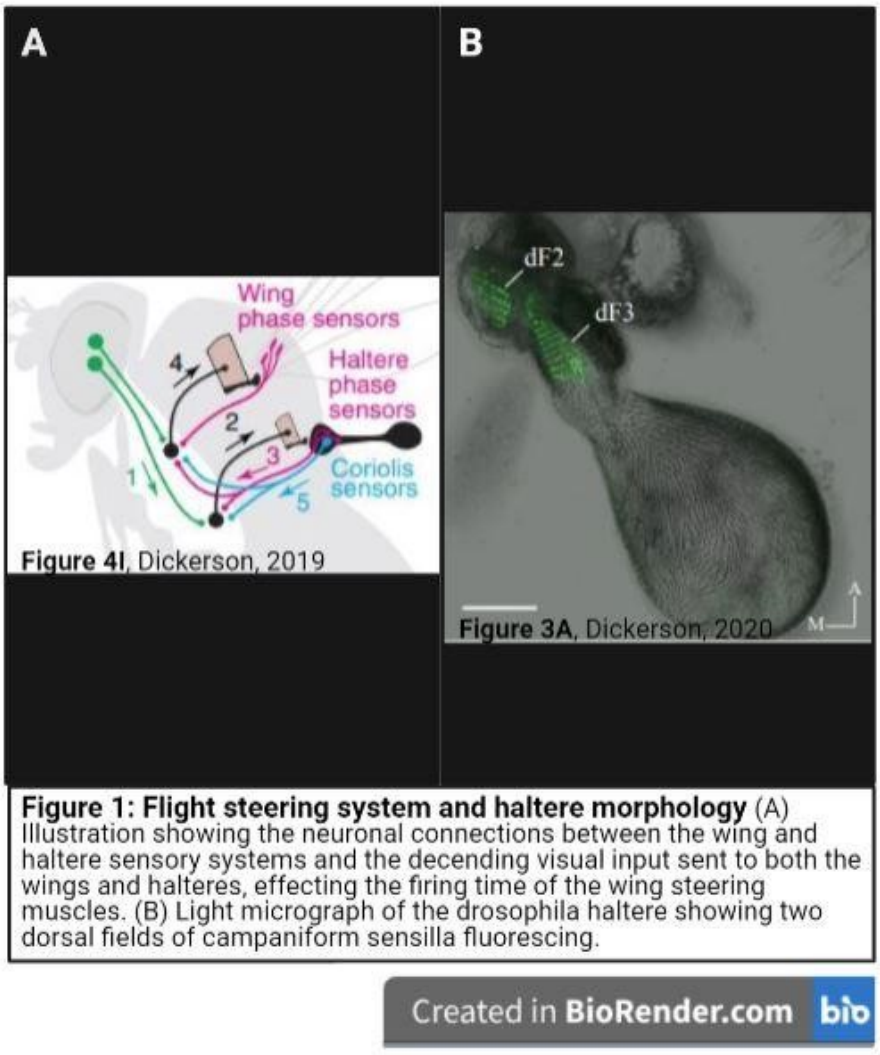
Introduction

The study of animal locomotion is a diverse field encompassing an enormous variety of intricate types of movement and motility. Animal flight is a particularly intriguing area of study because of the many complex processes at play during flight, such as timing of motor commands, control of limb kinematics, and the production of aerodynamic forces. *Drosophila melanogaster* are excellent organisms for studying animal flight due to their small size, short lifecycle, and streamlined physiology. These features make them a popular model used to better understand more complex flight processes in other organisms. However, *Drosophila's* simple yet organized physiology is impressive in its own right due to its exceptionally high efficiency. *Drosophila* only weigh about 10 μN, but are able to beat their wings at about 200 beats per second (Dickinson & Götz, 1996). They are also skilled flyers that can perform high-speed escape maneuvers (Muijres et al., 2014).

Drosophila are of the order Diptera and, thus, do not have hindwings. Instead, Diptera have small appendages called halteres, which evolved from hindwings. The halteres are present on the thorax of Diptera and beat antiphase to the wings (Pringle, 1948). The surfaces of halteres are covered with microscopic sensors called campaniform sensilla, which provide mechanosensory feedback that is sent to the wing steering muscles (Fayyazuddin & Dickinson, 1996, Fig. 1B). One major role for the halteres is their function as gyroscopes, helping to detect different types of body motion (Pringle, 1948). A paper by Nalbach (1993) analyzed the trajectory of the haltere in blowflies and how this trajectory allows different inertial forces to act on the halteres. Nalbach found that, rather than simply beating up and down, halteres actually

beat in a slightly triangular shape (1993). As a result, the bulb of the haltere is particularly sensitive to inertial forces such as the Coriolis force, which acts in a rotating frame of reference, altering the haltere's trajectory during body rotations (Nalbach, 1993).

Although the haltere acts as a passive sensor of external forces, it is also under active control. Like the wing muscles, haltere muscles have been shown to respond to visual input (Chan et al., 1998; **Fig. 1A**). In what is known as the control-loop hypothesis, Chan et al. suggested that flies might control voluntary turns by manipulating the trajectory of the halteres while they are beating so that they experience the same type of inertial forces they would during an involuntary movement, driving the same timing changes in wing-muscle activation (1998).



Recent evidence confirms the control-loop hypothesis as a plausible mechanism for initiating maneuvers. Visual stimulation alone changes the beating of the halteres, and in turn, the mechanosensory feedback from their embedded campaniform sensilla (Dickerson et al., 2019). Additionally, activation of the haltere steering-muscle motor neurons alters the firing time or recruitment of the wing steering muscles (Dickerson et al., 2019). The phase of the wing b1 muscle, in particular, seems to be strongly influenced by haltere stimulation (Fayyazuddin & Dickinson, 1996; Dickerson et al. 2019). Thus, for both voluntary and involuntary steering, changes in haltere motion are a critical signal to the wing steering system. Exactly how the small steering muscles at the base of the haltere are able to generate these trajectory changes, however, is not yet well understood.

Halteres are controlled by both a large power muscle and smaller steering muscles (Dickerson et al., 2019). Because of *Drosophila's* high wing and haltere beating frequency, the haltere power muscle is activated and contracts asynchronously to the beating of the wings and halteres (Lindsay et al., 2017; Dickinson & Tu, 1997). This allows the power muscle enough time to fully contract and relax, generating the force required to beat the halteres up and down (Lindsay et al., 2017; Dickinson & Tu, 1997). The steering muscles, on the other hand, produce less power as they fire synchronously to the beating of the halteres, but are more important for controlling small, precise movements (Lindsay et al., 2017). Each steering muscle is controlled by one motor neuron (Trimarchi & Schneiderman, 1994). The haltere steering muscles are divided into the basalars and the axillaries, with the basalares consisting of hB1 and hB2 and the axillaries consisting of hI1, hI2, hIII1, hIII2, and hIII3 (Dickerson et al., 2019). Though the muscles of the wing steering system are not identical to those of the haltere, they are serially homologous. The wing steering muscles have been shown to be either tonically or phasically

active during flight (Lindsay et al., 2017). The motor neurons of the tonically active muscles fire with every beat of the stroke cycle at a precise time, or phase, and seem to be important for making small changes in movement during flight (Lindsay et al., 2017). By contrast, the motor neurons of the phasically active muscles fire in short bursts, with each action potential still taking place at a particular time in the stroke cycle, indicating an important role in generating large turns and motions called body saccades during flight (Lindsay et al., 2017). The phasically active wing muscles were found to be b2, i1, iii1, hg1, and hg3, and the tonically active wing muscles were found to be b1, b3, i2, iii3, and hg4 (Lindsay et al., 2017). Though it has not yet been determined which of the haltere steering muscles are either phasically or tonically active, it is likely that they show a similar pattern to the wing muscles.

To understand how phase changes in the activation of the muscle motor neurons can influence how the muscles function, it is important to first understand what factors affect the amount of force a muscle generates. Robert K. Josephson published a paper in 1985 explaining how the work output of a given muscle undergoing cyclic length changes can be measured by plotting the force of a muscle against its length, while the muscle is both lengthening and shortening. His research showed that subtracting the work needed for the muscle to lengthen from the work generated while the muscle is shortening reveals the work output of the muscle (Josephson, 1985). Therefore, the time during the stroke cycle at which a *Drosophila* wing steering muscle is activated can affect the force output of the muscle and how it functions during locomotion.

The haltere muscles attach to sclerites via tendon-like structures called apodemes, which are critical for helping to transmit the mechanical power generated by the muscles to the hinge of the haltere. However, more research is required to understand how each of the haltere steering

muscles is controlled during flight and how they signal timing information to the wing steering muscles. Understanding the functional anatomy of the haltere steering system and how it controls trajectory changes could help us to better understand the structure and physiology of the haltere hinge, and might also help us to understand the mechanisms behind the much larger trajectory changes generated by the wing hinge during flight. Yet, little is currently known about the number and location of the haltere apodemes.

In this study, I imaged and mapped the haltere apodemes by using confocal microscopy to image hemisected flies expressing GFP in their apodemes. I then captured videos of the apodemes fluorescing in a live fly during flight. The results indicated that the apodemes of the two basalar muscles (hB1 and hB2) are fused to one another, and that this fused apodeme, along with the apodeme of the hIII2 muscle, was particularly prominent.

Methods

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-GFP, rabbit polyclonal antibody, AlexaFluor 488 conjugate	Invitrogen/ThermoFisher Scientific	A-21311
Alexa Fluor 568 phalloidin	Invitrogen/ThermoFisher Scientific	A-12380
Chemicals, Peptides, and Recombinant Proteins		
Fixative Solution (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1mL NaCaCo, 0.04%CaCal2	From CORE	
Triton X-100	Sigma-Aldrich	T-8787
PBS, 10x solution pH 7.4	ThermoFisher Scientific	J75889-K2
alpha-Thioglycerol	TGI	S0374
Tissue-Tek O.C.T. compound	Sakura	4583
D-(-)-Fructose	Sigma	F-0127
Deposited Data		
Raw and analyzed data	This paper	
Experimental Models: Organisms/Strains		
D. melanogaster: UAS-mCD8-GFP; UAS-stingerRed	The Dickinson Lab at Cal Tech	
D. melanogaster: *short stop*	The Dickinson Lab at Cal Tech	
Software and Algorithms		
GIMP	https://www.gimp.org/	RRID:SCR_003182
FIJI	NIH (https://fiji.sc/)	RRID:SCR_002285

Experimental Model and Subject Details

Flies were provided by the Dickinson Lab at Caltech. They were used at 1-5 days old and raised at 25℃ on a 12:12 light/dark cycle. To create the cross used for mapping the apodemes, UAS-mCD8-GFP virgins, which had been bred to express GFP in their haltere apodemes, were crossed with short stop males. To create the cross used for imaging during flight, short stop virgins were crossed with UAS-mCD8-GFP males. Examining F1 flies from both crosses under a fluorescent microscope, flies that appeared to express the most GFP in their thoraxes were selected for use.

Method Details

Performing the hemisection

I performed the hemisection following the protocol developed by Anne Sustar from the Dickinson Lab (2014). Six F1 flies, consisting of 5 females and 1 male, were selected and anesthetized by cooling them to 4°C for 1 minute. The flies were then placed in a petri dish containing 95% ethanol for 3 minutes. After rinsing the flies twice in petri dishes of DI water, the flies were transferred to a petri dish containing PBS.

In PBS, I removed their wings and legs. Filling a mold with Optimal Cutting Temperature Compound (O.C.T.), the flies were placed dorsal-side up into the wells of the mold, 3 flies per well. Each well of the mold was approximately 3mm x 17mm and held approximately 100µL of O.C.T. The molds were frozen for 15-20 minutes. Once frozen, I sliced the flies embedded in the cubes of O.C.T. along their sagittal plane using an ultra-fine razor. The frozen pieces of O.C.T. containing the now hemisected flies were removed from the wells and transferred to a petri dish of fixative solution (4% formaldehyde in PBS) to allow the O.C.T. pieces to dissolve and the hemisections to be released.

Once dissolved, the hemisected samples were transferred to 0.6mL Eppendorf tubes of 0.4mL of the same fixative solution and nutated for 45 minutes in the dark. Afterwards, the samples were rinsed in PBS-Tx for three 20-minute intervals, nutating the samples continuously during each rinse cycle. Next, the samples were nutated in a phalloiden/GFP stain in the dark in a 4°C environment for 8 days. The stain consisted of a 1:50 ratio of Alexa Fluor 568 phalloidin (Invitrogen. Cat. No. A12380) and a 1:100 ratio of anti-GFP, rabbit polyclonal antibody, AlexaFluor 488 conjugate (Invitrogen. Cat No. A21311).

Preparing and imaging the samples

On day 8, the flies were imaged. To prepare for imaging, I rinsed the hemisected samples again in PBS-Tx for 15 minutes. The samples were then cleared in a 20%, 40%, 60%, 80%, and 100% concentration of a SeeDB clearing solution for 15 minutes each. The SeeDB solution contained fructose and 0.025 mL alpha-thioglycerol (TCI Chemicals, Catalog No. S0374). After clearing, I mounted samples cuticle-side up using an 80.2% concentration of the SeeBD solution. The samples were then imaged with a 40x objective by Tony Perdue from the UNC Departmental Imaging Core Facility using a confocal microscope to create confocal stacks of the samples. Three rounds of hemisected flies were prepared and imaged over the course of three months.

Imaging the apodemes during flight

To image the apodemes during flight, I first tethered 2-5 day old flies with a tether glued to the dorsal side of their thorax. The tethered flies were then placed inside a flight arena displaying different patterns of LED light generated in a random order. Using a custom-built epifluorescence microscope from ThorLabs parts (Newton, NJ), I imaged the live flies using a 50x NA objective and Retiga R1 camera. The camera was triggered at a 0.05 phase relative to the upstroke of the wings and the exposure time of the camera was 21ms. A wing-beat analyzer placed below the fly measured behavioral data, such as wing-beat frequency and amplitude.

Quantification and Analysis

I uploaded a single confocal channel into GIMP software and added a blank layer between each existing layer of the stack. Using the raw confocal images as a guide, I traced the apodemes and haltere steering muscles onto the blank layers. I then deleted the layers with the confocal data and merged the traced layers creating a TIFF stack for each channel that I traced.

The TIFF stacks were then overlaid on the brightfield channel of their corresponding confocal image.

Results

At least six apodemes were identified

To understand how the haltere steering muscles generate these trajectory changes, it is important to understand how the steering muscles at the hinge of the haltere generate force and how they transmit this force to the halteres. The steering muscles attach to the halteres via tendon-like structures called apodemes, but the location and number of these apodemes was previously unknown. By creating fly hemisections and staining the steering muscles and apodemes with phalloidin and GFP respectively, I was able to image the apodemes (**Fig. 2A-B**). I then used GIMP software to trace the apodemes (**Fig. 2C**).

Because there are seven haltere steering muscles, I expected to find at least seven apodemes. In my first tracing, I identified six apodemes or apodemes clusters. Using FIJI/ImageJ, I then measured the two-dimensional area of these tracings. I also determined their depth by counting how many confocal layers were traced for each apodeme in GIMP.

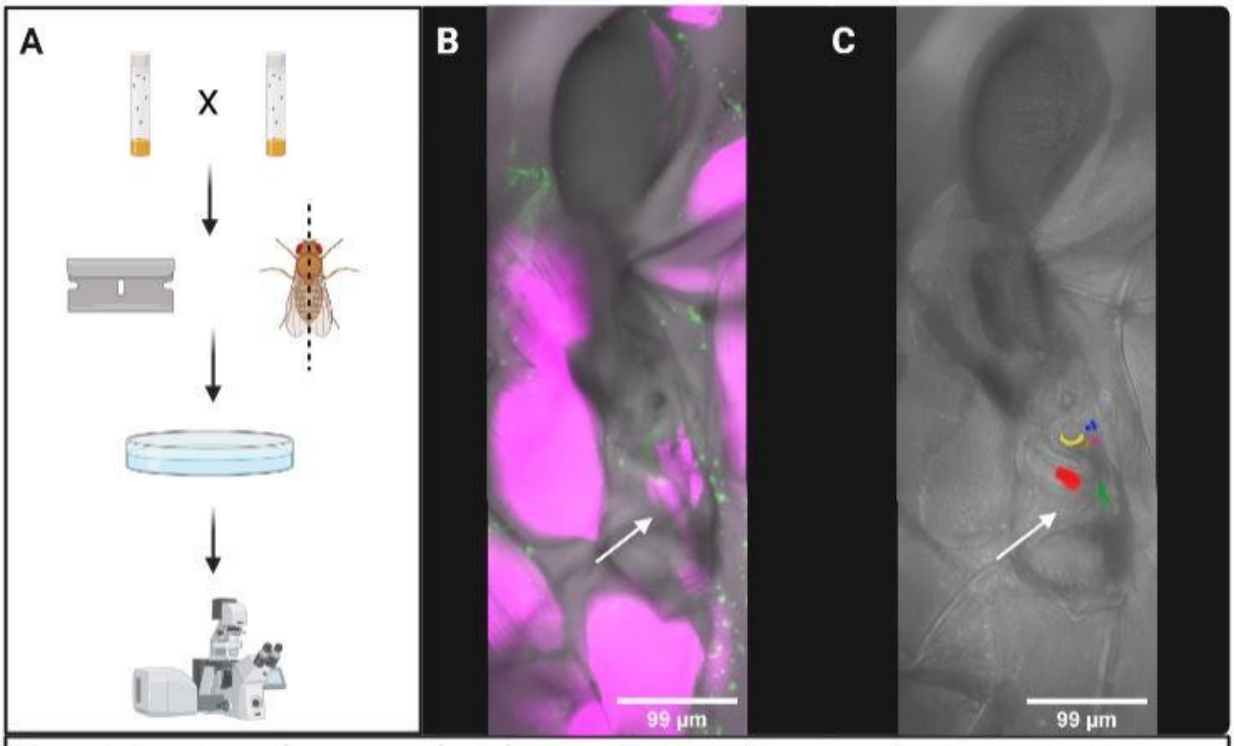


Figure 2: Experimental process and apodeme tracings (A) Schematic showing the steps of the experimental process including the cross, the hemisection, the staining and the confocal imaging. (B) Average stack created from a confocal image showing the basic anatomy of the *Drosophila melanogaster* haltere, with its haltere steering muscles (magenta) and haltere apodemes (green) visible inside. (C) Bright field max stack of a confocal image of a haltere, overlaid with tracings of the haltere apodemes.

The basalar apodemes appear to be fused to one another

I completed a second tracing using another image to analyze the apodemes from a slightly different angle. In my second tracing I again identified six apodemes or apodeme

Apodeme Identifying Color and Corresponding Muscle	Mean Apodeme 2D Area (microns^2)	Mean Apodeme Depth (microns)	Estimated Apodeme Volume (microns^3)
Red (hB1 & hB2)	210	12.5	2625
Green (hIII2)	183	14.5	2653.5
Yellow (hI2)	77.5	6.5	503.75
Blue (hIII1)	76	14.5	1102
Pink (hIII3)	75	10.5	787.5
Orange (hI1)	36.5	7	255.5

Table 1: For the two tracings, the 2-dimensional area of each apodeme was measured in microns^2 and the depth of each apodeme was measured in microns. The mean values were calculated for each apodeme and reported above. These values were then used to calculate the estimated volumes of each apodeme.

clusters (**Fig. 3A**). Using the same image, I then traced the haltere steering muscles themselves. To identify the steering muscles, I used a previous tracing of these muscles by Dickerson (2020) as a guide. I attempted to identify which apodemes attach to which steering muscles by referring to my raw image. In doing so, I was able to establish which apodemes connect to each of the seven steering muscles (**Fig. 3B**).

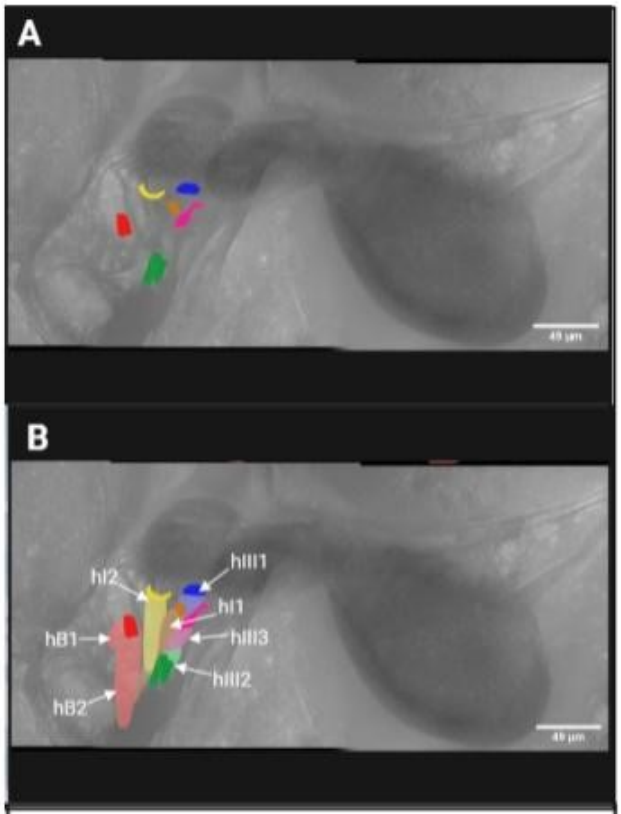


Figure 3: Comparing the apodemes with their corresponding steering muscles (A) Confocal brightfield stack of a haltere overlayed with tracings of the haltere apodemes. (B) Confocal brightfield stack overlaid with tracings of the haltere apodemes, as well as their six corresponding haltere steering muscles.

Furthermore, I observed that the apodemes of the two basalar muscles (hB1 & hB2) appear to be fused to one another and can be seen as one mass in my tracing (red) (**Fig. 3B**). I will from now on refer to this as the basalar apodeme. As before, I then measured the two-dimensional area of the apodeme tracings as well as their depth. I calculated the mean area and depth values for each apodeme and reported them in **Table 1**. I also used the mean area and depth measurements to calculate the estimated volume for each apodeme (**Table 1**). In doing so, two apodemes stood out as significantly larger than the rest (the basalar apodeme and the hIII2 apodeme) and the other four appeared smaller (hI2, hIII1, hIII3, and hI1). For all of the apodemes, the area and depth measurements varied somewhat between my two tracings, but this was particularly the case for the smaller apodemes (hI2, hIII1, hIII3, and hI1). I attributed this variance to the fact that the apodemes were traced

from slightly different angles in each of the images. Additionally, as these four apodemes were considerably smaller than the other two, they were more difficult to visualize and trace, making my measurements of their size less reliable. Therefore, I decided to focus my attention on the larger basalar and hIII2 apodemes.

Imaging the apodemes during flight did not provide conclusive results

Having successfully mapped the apodemes, I attempted to image the apodemes during flight to understand how the haltere steering muscles undergo length changes. To do this, I imaged a tethered fly in a flight arena with an epifluorescence microscope and a QImaging camera (**Fig. 4A**). If I was able to visualize one or more of the apodemes fluorescing, movement of this spot of fluorescence during flight would be an indication of the corresponding haltere steering muscle undergoing length changes. I identified the basalar apodeme clearly fluorescing in my footage and decided to use this apodeme as my guide. Though I was able to clearly visualize this apodeme (**Fig. 4B**), my fly sample population proved to be problematic and reluctant to fly. Unfortunately, time constraints prevented me from generating a new cross and population. Nonetheless, I was able to capture footage containing some sporadic bursts of flight (**Fig. 4C**). The apodeme of the haltere spiracle (an opening used for gas exchange) was also visibly fluorescing in my footage (**Fig. 4B**). I used this footage to create a kymograph showing how the position of the basalar apodeme changed during periods of flight (**Fig. 4D**). The apodeme did appear to move somewhat during flight, but clearer data is required before any conclusions can be made with confidence.

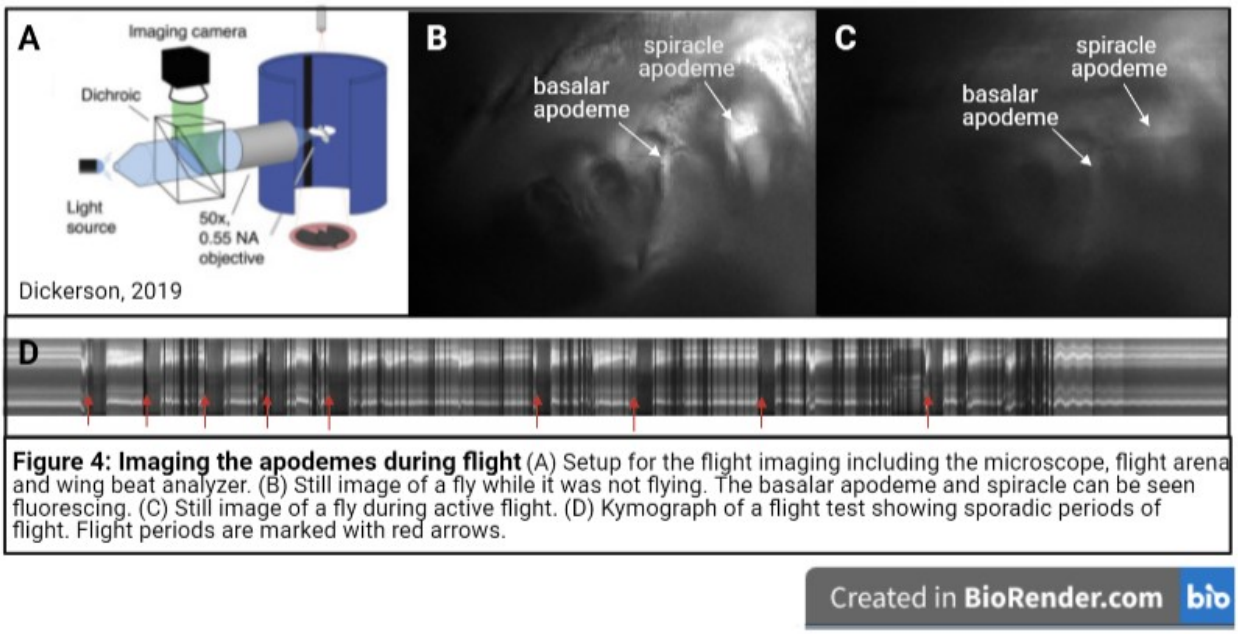


Figure 4: Imaging the apodemes during flight (A) Setup for the flight imaging including the microscope, flight arena and wing beat analyzer. (B) Still image of a fly while it was not flying. The basalar apodeme and spiracle can be seen fluorescing. (C) Still image of a fly during active flight. (D) Kymograph of a flight test showing sporadic periods of flight. Flight periods are marked with red arrows.

Discussion

My tracings showed that the largest apodemes of the haltere steering muscles appear to be the basalar apodeme and the apodeme of the axillary muscle hIII2. These two apodemes stood out as having a significantly larger estimated volume than the other four apodemes. Furthermore, the apodemes of the two basalar muscles (hB1 &hB2) appeared indistinguishable and therefore were considered to be one fused apodeme. My footage of the basalar apodeme during flight showed some possible movement of the apodeme, but these data were inconclusive.

These findings present two paths of inquiry: how the size of the apodemes relates to the size of their corresponding muscles and what conclusions can be drawn from the fact that the basalar muscles share an apodeme. The first question can be answered by looking at the size of the steering muscles. It is known that the cross-sectional area of a muscle is proportional to the amount of force it is capable of generating (Biewener & Patek, 2018). Because the hB2 muscle is very large, and the fact that both it and the hB1 muscle connect to the same apodeme, it is unsurprising that this apodeme is one of the largest, as it would likely be under a lot of strain.

However, the hIII2 is one of the smallest of the steering muscles, so it is both intriguing and puzzling that its apodeme is quite large.

The size of the apodemes and their corresponding muscles may also indicate their importance in influencing the timing of the wing steering muscles. Previous research has shown that the hB1 haltere muscle is recruited by a very large motor neuron with a thick axon (Sustar, n.d.; **Fig. 5**). The cross-sectional diameter of a motor neuron is indicative of the speed with which it conducts signals (Purves et al., 2001). Therefore, one explanation for the large size of the hB1 motor neuron is that this muscle may be tonically active. Tonically active muscles fire with every wing/haltere stroke and, thus, have to be recruited very quickly (Lindsay et al., 2017). Furthermore, since both the hB1 and hB2 muscles share an apodeme, the hB2 muscle may be recruited in tandem with the hB1 muscle and may also be particularly important for influencing timing. This fused basalar apodeme connecting to the two basalar muscles has also been found to exist in the haltere of other species of flies: horseflies (Bonhag, 1948) and blowflies (Chan et al., 1998). This indicates that this anatomy seems to be homologous among different species of flies. Additionally, earlier research on blowflies has shown that the firing timing of the first-wing basalar muscle (wb1) is particularly influenced by input from the halteres (Fayyazuddin & Dickinson, 1996; Dickerson et al., 2019). Therefore, the serially

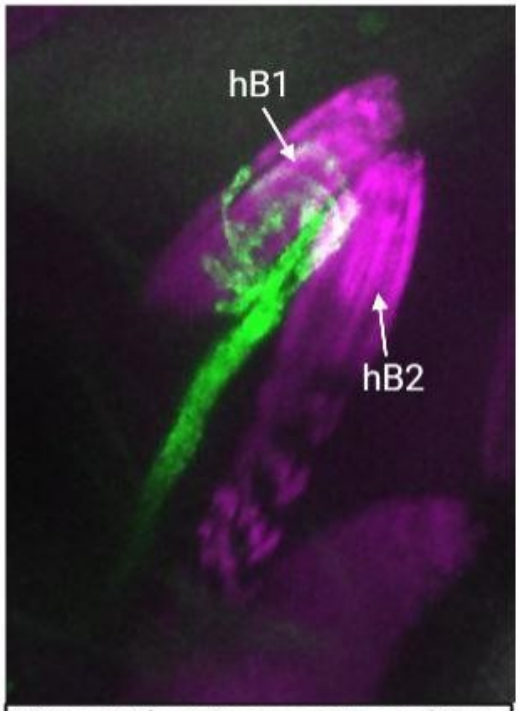


Figure 5: Above image courtesy of Anne Sustar The two haltere basalar muscles hB1 and hB2 can be seen in magenta. A large motor neuron (green) can be seen innervating the hB1 muscle.

homologous haltere basalar muscles (hB1 & hB2) may be particularly important for controlling haltere-induced changes in the wing muscles. Though not possible with our data, comparing the sizes of all of the haltere apodemes and steering muscles with those of the wing steering system would be another interesting way of exploring the possible functions of each haltere steering muscle.

Though I was unable to obtain clear footage of the haltere apodemes during flight, my near-successful attempt demonstrated that imaging the apodemes during flight is possible and paves the way for future research in this area. Definitive footage will help us to better understand what length changes the steering muscles undergo during flight and how exactly such a small group of muscles are able to generate precise trajectory changes during flight.

My findings may also provide a better understanding of the mechanics of the haltere hinge. Previous research has shown that, while the halteres and wings are connected through the nervous system, they are also mechanically connected, helping them to beat synchronously though antiphase (Deora et al., 2014). The mechanical linkage exists in an area on the thorax called the subepimeral ridge, keeping the wings and halteres in phase with one another (Deora et al., 2014). However, a mechanical clutch at the base of the wings allows the wings to either connect or disconnect from this mechanical linkage, allowing the fly to move its wings independently of one another if needed (Deora et al., 2014). Mapping the locations of the haltere apodemes may give us a clearer understanding of these mechanical connections, and builds the groundwork for new inquiries and research into how this morphology affects the kinematics of Dipteran flight.

Acknowledgements

I would like to thank all the members of my lab, but particularly my PI, Brad Dickerson, for all of his guidance and support in helping me with this research and with the writing and editing of my thesis. I would like to thank my lab manager, Lilly Price, for all her help with my experiments, maintaining my fly crosses, and helping to edit this thesis. And I would like to thank Annelise Marsh who helped to skillfully tether the flies for my flight experiments. I would like to thank Tony Perdue, from the UNC Departmental Imaging Core Facility, for imaging my samples, and Anne Sustar, from Caltech, for providing the hemisection protocol and haltere motor neuron image used in this thesis. I would also especially like to thank Dr. Amy Shaub Maddox and Jeanne-Marie McPherson, as well as my group partners from my BIOL 692H class, for all of their guidance and advice in editing my thesis and helping me to create this talk.

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